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TNF production by the medullary thick ascending limb of Henle's loop

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TNF production by the medullary thick ascending limb of Henle's loop. Medullary thick ascending limb of Henle's loop (mTALH) tubules, isolated from kidneys of male Sprague-Dawley rats, expressed the gene for tumor necrosis factor (TNF) and released this cytokine when challenged with lipopolysaccharide (LPS). The TNF produced was biologically active, as determined by cytotoxic activity present in supernatants from LPS-stimulated mTALH, using the TNF-sensitive murine fibrosarcoma cell line, WEHI 164. The amount of TNF produced, approximately 75 nM, has previously been shown to affect ion transport in the mTALH. The TNF-mediated cytotoxicity (and ion transport effects) were completely neutralized with a polyclonal anti-TNF antisera. Further, immunoprecipitation experiments demonstrated that the 17 kDa TNF monomer was formed by *de novo* protein synthesis. In contrast, the mTALH did not produce the related cytokine, lymphotoxin (LT). Production of TNF was confirmed by demonstrating the accumulation of a 1.6 kb TNF mRNA by Northern blot analysis; mRNA for LT was not detected. Expression of the TNF gene in the mTALH was confirmed by the polymerase chain reaction (PCR). Southern blot analysis and ethidium bromide staining of the resultant PCR products revealed the expected 276 bp sequence of TNF DNA for the mTALH. We have demonstrated that mTALH tubules stimulated with LPS express the gene for TNF, but not LT, and release biologically active TNF. TNF is an important mediator of septic shock and may contribute to changes in renal function associated with endotoxemia. Production of TNF by the mTALH may be an important autocrine regulatory mechanism for this nephron segment.

Tumor necrosis factor-alpha (TNF) is a cytokine that has both protective and pathophysiological effects *in vivo*. For example, TNF has a protective role in bacterial and parasitic infections, but contributes to the immune and inflammatory events associated with graft versus host reactions, experimental allergic encephalomyelitis, delayed-type hypersensitivity reactions, and autoimmune diseases [1]. TNF has also been implicated as an important mediator of cachexia and endotoxemia. Although macrophages and T cells are the major cellular sources of TNF, there is increasing evidence of TNF production by non-immune cells. For example, renal production of TNF has been demonstrated in proximal tubules [2] and mesangial cells [3], and astrocytes also have been shown to produce TNF [4]. Further, lipopolysaccharide (LPS)-stimulated proximal tubular epithelial cells express both the membrane-associated and secreted forms of TNF [5].

TNF mRNA expression increased to a greater extent in the kidney, compared to other organs, after *in vivo* administration of endotoxin [6]. Further, increased release of TNF, and a positive correlation in serum concentrations of TNF to blood urea nitrogen and creatinine levels, were observed in an *in vivo* renal ischemia/reperfusion injury model [7]. The resultant decrease in renal function was attributed to increased TNF production by infiltrating neutrophils during the reperfusion period. However, it is conceivable that both infiltrating immune cells and renal cells could have contributed to the observed effects by producing TNF.

The medullary thick ascending limb of Henle's loop (mTALH) transports 25% of the filtered load in the kidney. It also regulates medullary interstitial osmolality, which is essential for the preservation of the renal counter-current exchange system and the ability of the kidney to produce either a hyperosmotic or hypo-osmotic urine. Thus, changes in the integrity/function of this tubular segment during inflammation could have significant effects on regulation of blood pressure and extracellular fluid volume. The mTALH also may be an important renal immunoregulatory site since cytokines, such as TNF and IL-1, bind to the Tamm-Horsfall glycoprotein, an immunosuppressive cell-specific marker present on the apical and basolateral membranes of the mTALH [8]. Furthermore, cytokines such as TNF, IL-1, and interferon-gamma induce the expression of cell adhesion molecules on endothelial and tubular epithelial cells [9].

Renal cyclooxygenase metabolizes arachidonic acid (AA) to several metabolites, including prostaglandin E_2 (PGE_2), that affect renal blood flow, glomerular filtration rate, and ion transport in the kidney. Interestingly, cytokines can affect epithelial ion transport through the induction of AA metabolites [10, 11]. Further, addition of exogenous TNF to cultured mTALH cells inhibits ion transport by a prostaglandin-dependent mechanism, suggesting that TNF could act as a paracrine regulator of ion transport in the mTALH [12]. In this study, we demonstrated expression of the TNF gene, accumulation of TNF mRNA, and subsequent TNF production by the mTALH in response to LPS stimulation. Thus, TNF also may act in an autocrine manner to affect ion transport in this nephron segment.

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Methods

Animals

Male Sprague-Dawley rats (Charles River Lab, Wilmington, Massachusetts, USA) weighing 300 to 325 g (9 weeks old) were maintained on standard rat chow (Ralston-Purina Co., Chicago, Ilinois, USA) and given tap water *ad libitum*.

Reagents

Tissue culture media, collagenase, and streptavidin agarose beads were obtained from Gibco (Grand Island, New York, USA), chemicals were from Sigma (St. Louis, Missouri, USA). Guanidine isothiocyanate was purchased from Fluka (Ronkonkama, New York, USA), ^{35}S-methionine/^{35}S-cysteine and GeneScreen were from NEN Dupont (Boston, Massachusetts, USA), and biotinylated goat anti-rabbit IgG was obtained from Pierce (Rockford, Illinois, USA). AMPLIFY® and ^{14}C-methylated molecular mass markers were purchased from Amersham (Arlington Heights, Illinois, USA). The vector pGEM4 was obtained from Promega (Madison, Wisconsin, USA), and the GeneAmp® RNA PCR kit was purchased from Perkin Elmer Cetus (Norwalk, Connecticut, USA). Sense and anti-sense primers were obtained from Stratagene (La Jolla, California, USA).

Isolation of mTALH tubules

The mTALH were isolated as previously described [12–14]. Briefly, male Sprague-Dawley rats were anesthetized with an intraperitoneal injection of pentobarbital (0.65 mg/100 g body wt). The kidneys were perfused with sterile 0.9% saline, via retrograde perfusion of the aorta, and cut along the corticopapillary axis. The inner stripe of the outer medulla was excised, minced with a sterile blade, and incubated for 10 minutes at 37°C in a 0.75% collagenase solution gassed with 95% oxygen. The suspension was sedimented on ice, and the supernatant containing the crude suspension of tubules was collected. The collagenase digestion was repeated with the remaining undigested tissue. The combined supernatants were spun, resuspended in Hank's buffer, and filtered through a 52 μm nylon mesh (Fisher Scientific, Springfield, New Jersey, USA). The filtrate was discarded, and the tubules retained on the mesh were resuspended in Dulbecco's modified eagle's medium (DMEM; Gibco, Grand Island, New York). Combination of the perfusion and size exclusion steps was done to eliminate blood elements that produce TNF from the preparation [13, 14].

Immunohistochemical identification of the Tamm-Horsfall glycoprotein

Tubules were resuspended in 1 ml of Dulbecco's modified Eagle's medium (DMEM)-containing 10 mM HEPES (pH 7.4; Sigma), and goat anti-human uromucoid antiserum (1:50) (Tamm-Horsfall glycoprotein; Cappel), and incubated for 20 minutes on ice, washed twice with phosphate-buffered saline (PBS), and resuspended in 1 ml of PBS. Aliquots of the tubule suspensions were washed, and then incubated for 30 minutes at 4°C with affinity-purified donkey anti-goat IgG (1:250) labeled with fluorescein isothiocyanate (FITC). The tubules were washed, resuspended in DMEM, and examined by fluorescence microscopy. Control samples were incubated with whole goat serum, or FITC-labeled anti-goat IgG in the absence of goat anti-human uromucoid antiserum.

Alkaline phosphatase assay

Alkaline phosphatase activity was determined by measuring the hydrolysis of p-nitrophenylphosphate. Activity was assessed in tissue homogenates obtained from undigested outer medulla and isolated mTALH tubules prepared in 0.01 M Tris containing 0.25 M sucrose, pH 7.4. Liver and renal cortical homogenates were used as negative and positive controls, respectively. Preliminary kinetic experiments ensured that the formation of p-nitrophenol from p-nitrophenylphosphate by each tissue was linear for 30 minutes. An aliquot of homogenized tissue, containing 50 μg of protein, was incubated for 30 minutes at 37°C in a substrate buffer (pH 10.5) containing 0.1 M glycine and 1 mM $MgCl_2$. The reaction was stopped with 50 mM NaOH, and the absorbance was measured at 405 nm. Protein concentration was determined using the Bradford assay [15].

Oxygen consumption

The oxygen consumption of freshly isolated mTALH tubules resuspended in DMEM was measured with a Clark-type electrode (Instech, Plymouth Meeting, Pennsylvania) inserted into a 0.3 ml temperature controlled (37°C) chamber (YSI, Yellow Springs, Ohio, USA). The amount of tubules (protein) added was titrated so that all the oxygen in the chamber was consumed in 30 minutes. The change in oxygen concentration in the chamber was monitored on a Soltec recorder. A constant slope was established during a control period, and additions to the chamber were made in volumes that did not exceed 1% of the volume of the measuring chamber. The rate of oxygen consumption was determined from the slope of the recorded curve, by measuring its angle against the vehicle oxygen consumption slope and calculating the tangent [14]. Results are expressed as nmoles of oxygen utilized/min/mg of tissue.

Bioassay for TNF

TNF bioactivity was measured as previously described [16] using the WEHI-164 fibrosarcoma cell line, which is sensitive and specific to the cytotoxic effects of TNF and lymphotoxin (LT). Serial dilutions of the supernatants derived from the mTALH tubules (0.5 g wet wt) were incubated with 5×10^3 WEHI-164 cells in 96-well microtiter plates for 48 hours at 37°C/5% CO_2. Cell viability was determined by the addition of 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT), a tetrazolium ring-containing compound which is cleaved by dehydrogenases of active mitochondria. Five mg/ml MTT was added to each well and incubated for four hours at 37°C/5% CO_2. One hundred microliters of 0.04 N HCl in isopropanol was added to each well, and plates were read using a test wavelength of 570 nm and a reference wavelength of 630 nm. One unit of bioactivity is defined as the supernatant dilution at which 50% cytotoxicity is observed. Recombinant murine TNF was used as a positive control.

Immunoprecipitation

mTALH tubules (0.5 g wet wt) were incubated with or without LPS in methionine-free DMEM in the presence of 200 μCi/ml of ^{35}S-methionine/^{35}S-cysteine for 20 hours. Samples were spun and the pellets containing the tubules were lysed in

PBS containing 1% Triton X-100 and 0.1% BSA. The lysed tubules, and the supernatants were pre-cleared with preimmune rabbit sera for one hour on ice. Samples were then incubated for two hours with biotinylated goat anti-rabbit IgG (50 μg/ml). Samples were washed and streptavidin agarose beads were added to the samples, which were mixed by inversion for 30 minutes. Samples were then spun and the supernatants were incubated for two hours with a 1:50 dilution of a rabbit polyclonal antisera against murine TNF [16]. These antisera inhibited the cytotoxic effects of mouse and human rTNF, and also inhibited TNF and LT produced by the murine T cell clone, F1.28. Antibody-antigen complexes were precipitated for two hours with biotinylated goat anti-rabbit IgG and streptavidin agarose as the solid matrix. The immunoprecipitates were washed with 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 0.1% ovalbumin in PBS and electrophoresed on a 15% acrylamide SDS-PAGE gel. The gels were treated with AMPLIFY®, dried by vacuum and exposed to Kodak X-Omat film with intensifier screens at −70°C. ^{14}C-methylated molecular mass markers were used as a standard and included the following proteins: myosin (200 kDa), phosphorylase b (97.4 kDa), BSA (69 kDa), ovalbumin (46 kDa), carbonic anhydrase (30 kDa), and lysozyme (14.3 kDa).

cDNA probes

A 1.4 kB murine TNF cDNA probe (a gift from Dr. Bruce Beutler, University of Texas Southwestern Medical School, Dallas, Texas, USA) was excised from the *BamHI* and *PstI* sites of the vector pGEM4. A 0.71 kB *KpnI/HincII* fragment of the murine LT cDNA was used as the LT probe [17].

Isolation of total RNA/Northern blot analysis

Total RNA was isolated by lysing the tubules in guanidine isothiocyanate/Na-citrate buffer followed by ethanol precipitation. Briefly, cells were lysed with guanidine isothiocyanate, passed vigorously through a 27 gauge needle, and incubated for 30 minutes with 100% ethanol (0.5 vol). Samples were spun at 12,000 × g, 4°C, supernatants were aspirated and the pellets resuspended in guanidine isothiocyanate. RNA was precipitated with ice-cold 100% ethanol and the pellets were successively washed with 70% ethanol and 100% ethanol. RNA samples were stored at −70°C as ethanol precipitates. Ten micrograms of total total RNA was electrophoresed in a 1% agarose/formaldehyde gel in 1× MOPS as the running buffer, as previously described [18]. RNA was then transferred to a nylon membrane (GeneScreen) and hybridized to random-primed ^{32}P-labeled probes in a buffer containing 50% formamide, 10% dextran sulfate, 0.2% polyvinylpyrrolidone, 0.2% Ficoll, 0.2% BSA, 1.0 M NaCl, 1.0% SDS, 0.05 M Tris pH 7.5, and 0.1% sodium pyrophosphate at 4°C for 18 hours. Post-hybridization washes of the membrane were carried out in 2× SSC, 1.0% SDS at 65°C for one hour and in 0.1× SSC at 25°C for one hour. Then the probed blots were exposed at −70°C to Kodak X-Omat film.

Polymerase chain reaction

A 1 μg aliquot of total RNA isolated from unstimulated or LPS-stimulated mTALH tubules was used for cDNA synthesis using the GeneAmp RNA PCR kit in a 20 μl reaction mixture containing cloned Moloney Murine Leukemia Virus reverse transcriptase (2.5 U/μl), RNase inhibitor (1 U/μl), and random hexamers (2.5 μM). The reaction mixture was incubated at room temperature for 10 minutes to allow extension of the primers by reverse transcriptase, and then at 42°C for 15 minutes, 99°C for five minutes, and 4°C for five minutes. An aliquot of the cDNA was then amplified using *Amplitaq* DNA polymerase (0.025 U/μl) in the presence of the sense primer (+412 to +432; +177; ATGAGCACAGAAAGCATGATC) and the antisense primer (+412 to +432; TACAGGCTTGTCACTCGAATT) for mouse TNF which were used at a final concentration of 1 μM. The amplification (30 cycles) was initiated by one minute of denaturization at 94°C, two minutes of annealing at 58°C, and polymerization for three minutes at 72°C followed by autoextension at 72°C for five minutes. The amplified product was then analyzed by Southern blot analysis following electrophoresis through a 1.5% agarose gel, transfer to nitrocellulose, hybridization with a ^{32}P-labeled cDNA probe for TNF and exposure to Kodak X-Omat film at −70°C.

Results

Characteristics of freshly isolated mTALH tubules

Rat mTALH tubules were isolated by excision of the inner stripe of the outer medulla, followed by enzymatic digestion and sieving [12–14]. This combination of techniques yielded a homogeneous (>95%) tubular suspension with long and well-defined tubules, which expressed the Tamm-Horsfall glycoprotein (Fig. 1). Little or no fluorescence was observed in tubules stained with whole goat serum and FITC-labeled rabbit anti-goat IgG, or with FITC-labeled rabbit anti-goat IgG added in the absence of goat anti-human Tamm-Horsfall glycoprotein. These data indicate that non-specific staining was minimal for the reagents used in these experiments.

TNF production by proximal tubules has been reported [2, 19]. Therefore, we measured alkaline phosphatase activity, a membrane marker of proximal tubules, to assess proximal tubule contamination as a possible source of TNF production. Alkaline phosphatase activity of the isolated mTALH was ten times less than for renal cortical homogenates (0.37 ± 0.09 vs. 3.55 ± 0.28 nmol/ml/min, respectively; Fig. 2). The activities of medullary and liver homogenates were 0.83 ± 0.27 versus of 0.22 ± 0.02 nmol/ml/min, respectively. Thus, activity of the isolated mTALH was similar to that of the liver homogenates (negative control). This suggests that the presence of proximal tubules was negligible, and confirms previous assessments of mTALH purity [12].

The Na^+-K^+-ATPase accounts for approximately 40 to 50% of the oxygen consumption by the mTALH [20]. Since high Na^+-K^+-ATPase activity in the mTALH is characteristic of this nephron segment, ouabain-sensitive oxygen consumption was assessed in the mTALH tubule preparation to determine if this functional parameter was intact. Addition of ouabain (1 mM), and determination of the slope of oxygen consumption revealed approximately a 42% decrease in oxygen consumption, indicating that an important mechanism regulating ion transport was retained by the freshly isolated tubules (Fig. 3A). The functionality of the Na^+-K^+-$2Cl^-$ cotransporter, a specific marker for the mTALH, also was assessed. Thus, addition of furosemide (0.1 mM), an inhibitor of the cotransporter, decreased oxygen consumption by approximately 29%. These data are similar to

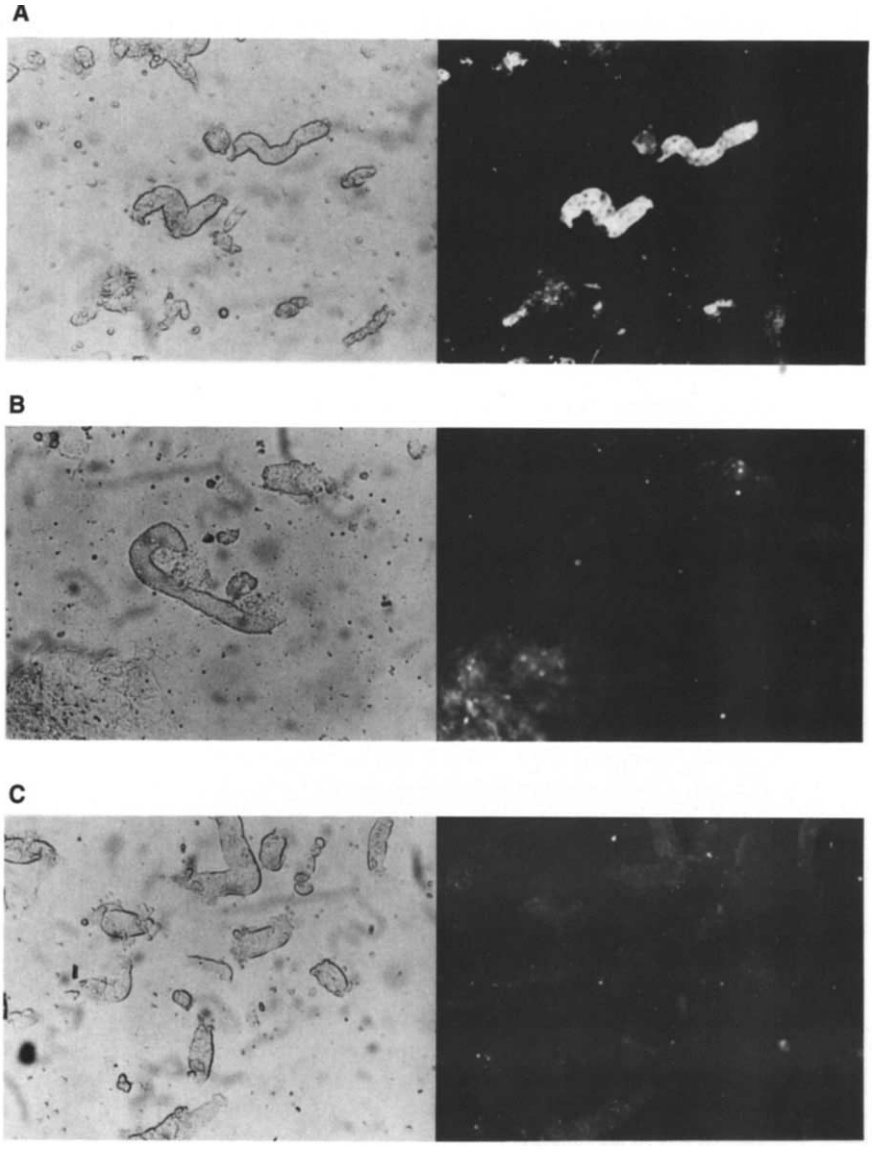

Fig. 1. *Immunofluorescent staining of the Tamm-Horsfall glycoprotein.* (**A**) mTALH tubules were incubated with goat anti-human uromucoid antiserum (Tamm-Horsfall glycoprotein) for 20 minutes on ice. The tubule suspensions were washed, and then incubated for 30 minutes at 4°C with FITC-labeled, affinity-purified donkey anti-goat IgG. The tubules were washed and resuspended with DMEM, and examined by light (left panels) and fluorescence (right panels) microscopy (Magnification 100×). Control samples were incubated with (**B**) whole goat serum and FITC-labeled anti-goat IgG, or (**C**) FITC-labeled anti-goat IgG in the absence of the primary antibody.

those previously reported [14], and suggest that the Na^+-K^+-$2Cl^-$ cotransporter was functional in the freshly isolated mTALH tubule preparation (Fig. 3B).

TNF production by mTALH tubules

Tubules were incubated for 3, 6, 9, and 24 hours in the absence or presence of 1 μg/ml LPS, and TNF bioactivity was measured using cell-free supernatants that were incubated with WEHI-164 cells for 48 hours. Cytotoxicity, indicative of TNF and/or LT activity, was observed at each of the time points and was maximal at 24 hours. Serial dilutions of the 24 hour supernatants showed significantly higher cytotoxic activity in the LPS-stimulated mTALH compared to unstimulated mTALH (Fig. 4). Additionally, the concentration of TNF was estimated to be approximately 75 nM, by extrapolating from a standard curve constructed using recombinant murine TNF. It should be noted that TNF production by cultured mTALH cells was estimated to be 5 nM [12]. Further, the levels of TNF production by LPS-stimulated mTALH tubules and cultured mTALH cells are in accordance with those shown to inhibit ion transport in the mTALH [12]. To verify that the observed cytotoxicity was TNF-mediated, serial dilutions of the supernatants were pre-incubated with a polyclonal anti-human TNF antibody prior to incubation with WEHI-164 cells. In the presence of antibody, cytotoxicity was completely eliminated (Fig. 5). Thus, the cytotoxic effects observed using supernatants from LPS-stimulated mTALH tubules were due to production of TNF and/or LT.

De novo synthesis of TNF

We metabolically radiolabeled the mTALH with ^{35}S-cysteine/^{35}S-methionine in the absence or presence of LPS, to ensure that the TNF detected by bioassay was synthesized and released by the mTALH, and was not released from TNF binding sites on the mTALH. The radiolabeled samples were immunoprecipitated using a polyclonal anti-TNF antibody and then separated by SDS-PAGE under reducing conditions. *De novo* synthesis of the 17 kDa TNF monomer was observed for

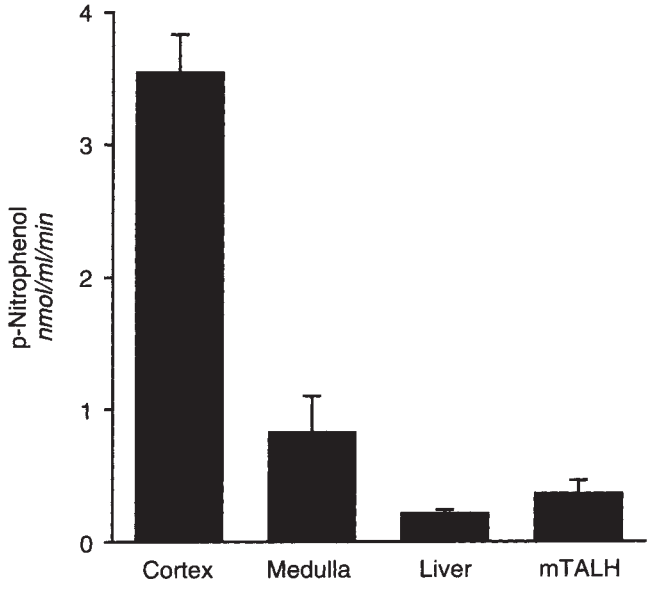

Fig. 2. *Alkaline phosphatase activity of the isolated mTALH versus renal cortical homogenates.* Alkaline phosphatase activity was determined spectrophotometrically by measuring the hydrolysis of p-nitrophenylphosphate. The activity of medullary tissue and liver homogenates served as negative controls. The data represent the mean ± SD of 4 separate experiments.

LPS-stimulated mTALH tubules, as well as supernatants from LPS-stimulated mTALH (Fig. 6). Smaller amounts of TNF were detected in the unstimulated mTALH. LT, which is structurally and functionally related to TNF, was not detected by immunoprecipitation experiments, suggesting that LPS-stimulated mTALH produced TNF, but not LT, by *de novo* protein synthesis.

Accumulation of TNF mRNA

Production of TNF by the mTALH was confirmed by demonstrating the presence of the mRNA for TNF by Northern blot analysis. Total RNA was isolated from mTALH tubules stimulated with LPS for 24 hours. RAW 264 cells, a murine macrophage cell line which produces TNF when stimulated with LPS, were used as a positive control. Accumulation of the 1.6 kb mRNA for TNF was observed in the RAW 264 cells and the LPS-stimulated mTALH, but not in the unstimulated mTALH (Fig. 7). The additional band observed for RAW 264 cells may be explained by several possible mechanisms including: a different start site for transcription, or differential mRNA splicing. We also determined if LT mRNA could be detected in LPS-stimulated mTALH tubules, since this lymphokine also has cytotoxic effects against the WEHI-164 target cell line. Northern blot analysis was performed using an LT cDNA probe on RNA extracted from mTALH tubules and from F1.28 cells, a T cell clone that produces LT when stimulated with anti-CD3 antibody. Accumulation of the 1.5 kB mRNA for LT was demonstrated for stimulated F1.28 cells, but not for RAW 264 cells or the mTALH (Fig. 8). These data demonstrate that the mTALH does not transcribe the gene for LT, and support the conclusion from immunoprecipitation experiments that the mTALH produces TNF, but not LT. Thus, all of the cytotoxic activity present in supernatants from LPS-stimulated mTALH, that was neutralized with anti-TNF antibody, was due to TNF.

TNF gene transcription

The presence of the TNF gene in LPS-stimulated mTALH tubules was determined using the polymerase chain reaction (PCR). The resultant PCR products were separated on a 1.5% agarose gel, transferred to nitrocellulose, and Southern blot analysis was performed using a ^{32}P-labeled TNF cDNA probe. Raw 264 cells were used as a positive control, and RNA from the pAW109 plasmid was used as a negative control, to ensure that the TNF cDNA probe recognized only sequences of the TNF gene. The 276 bp fragment of TNF DNA was observed for RAW 264 cells and mTALH, but not for pAW109, by Southern analysis, thus verifying the presence of the TNF gene in the mTALH (Fig. 9A). Ethidium bromide staining also revealed the presence of the predicted 276 bp fragment of TNF DNA for RAW 264 cells and the mTALH (Fig. 9B).

Discussion

We have demonstrated that the mTALH segment of the nephron can produce and release biologically active TNF in response to LPS stimulation. TNF bioactivity was assessed using the WEHI-164 bioassay, and cytotoxicity was significantly higher in LPS-stimulated compared to unstimulated mTALH. Furthermore, cytotoxicity was completely eliminated in the presence of a polyclonal anti-TNF antiserum, which can neutralize biologically active TNF and LT. Metabolically radiolabeled mTALH incubated in the presence of LPS produced TNF, but not LT, by *de novo* synthesis. These experiments were performed to ensure that the TNF detected by bioassay was synthesized by the mTALH, and to determine whether the mTALH produced LT, which cannot be distinguished from TNF in the bioassay. The 17 kDa TNF monomer was observed for lysed tubules and supernatants obtained from LPS-stimulated mTALH. Thus, the mTALH released biologically active TNF after it was produced and also may have expressed a membrane-bound form of TNF, as described for other cell types [21]. A lesser amount of TNF also was detected in the unstimulated mTALH. This may represent a low basal production of TNF or may be the result of minimal amounts of LPS contamination. In contrast, LT production was not detected by immunoprecipitation, suggesting that all of the cytotoxic activity present in supernatants from LPS-stimulated mTALH was due to TNF, and not LT. Transcription of the TNF gene in LPS-stimulated mTALH was confirmed by assessing the accumulation of mRNA for TNF by Northern blot analysis. The WEHI-164 cell line cannot differentiate between TNF and LT, nor is there an available antibody which can distinguish between the two. Thus, accumulation of mRNA for LT also was assessed. Accumulation of the 1.6 kb mRNA for TNF was observed in the LPS-stimulated mTALH and RAW 264 cells, but was not detected in the unstimulated mTALH. In contrast, Northern blot analysis using a cDNA probe for LT demonstrated accumulation of the 1.5 kB mRNA for LT in stimulated F1.28 cells (a T cell clone that is positive for LT when stimulated with anti-CD3 antibody), but not in the LPS-stimulated RAW 264 cells or the mTALH. Thus, the Northern blot analyses confirm the data from immunoprecipitation experiments, and support the conclusion that LPS-stimulated mTALH

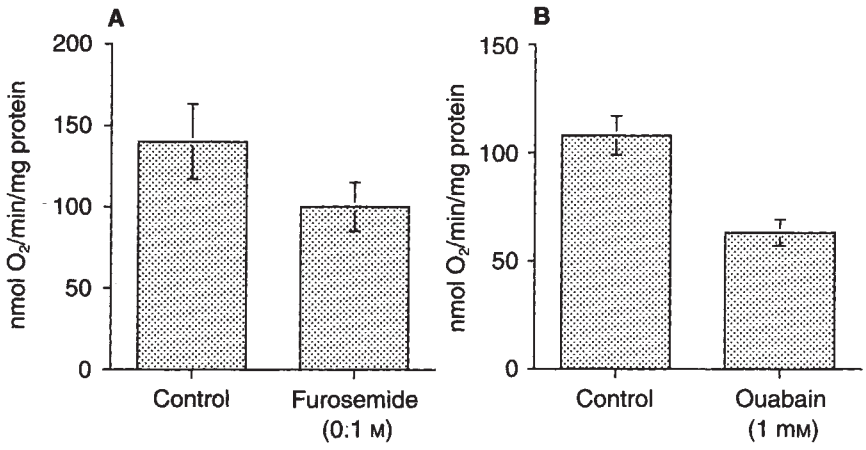

Fig. 3. *Oxygen consumption by mTALH tubules.* Isolated mTALH tubules were incubated with (**A**) ouabain (1 mM), or (**B**) furosemide (0.1 mM) at 37°C in a closed chamber, and oxygen content was monitored continuously as described in **Methods**. Oxygen consumption is expressed as nmol O_2 consumed/min/mg protein, and the data represent the mean + SE, n = 7. Data were analyzed by a paired Student's *t*-test. * $P <$ 0.01, ** $P < 0.001$.

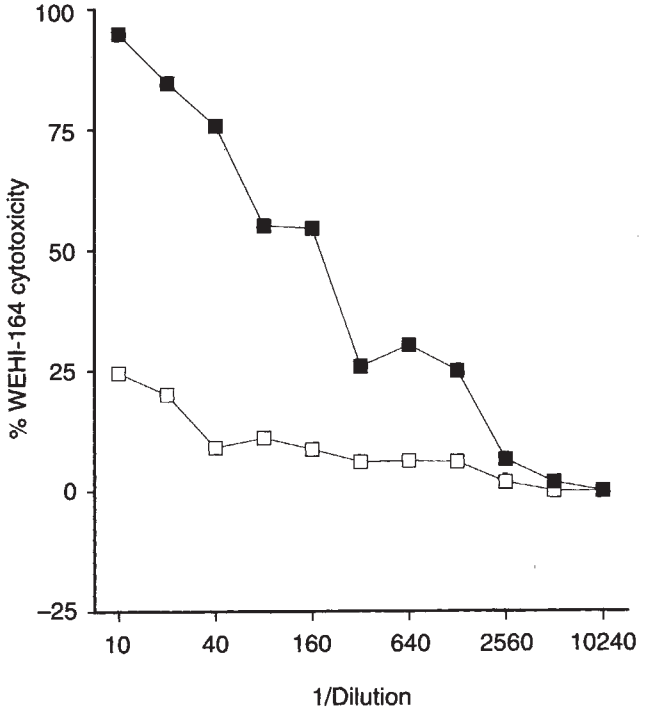

Fig. 4. *TNF production by the mTALH.* mTALH tubules were incubated in the presence (■) or absence (□) of LPS for 24 hours. Supernatants were harvested and TNF bioactivity was determined using the WEHI-164 cell line. The figure is representative of four similar experiments.

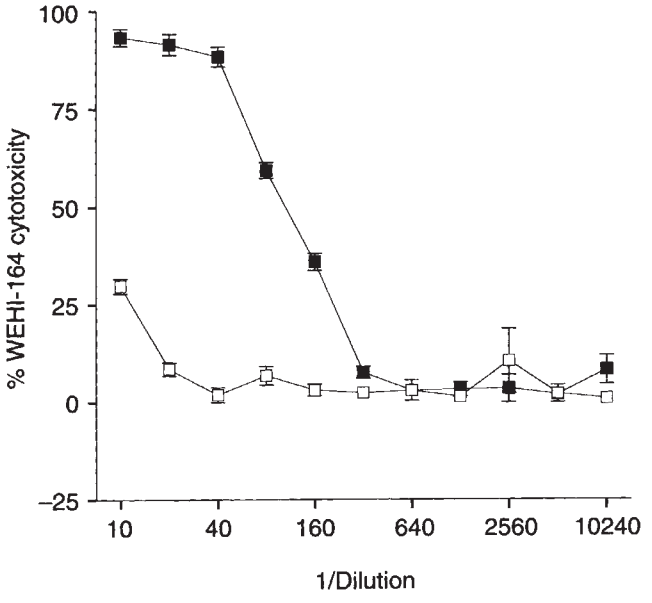

Fig. 5. *Neutralization of TNF bioactivity.* mTALH tubules were incubated with LPS for 24 hours. Supernatants were harvested and pre-incubated with either polyclonal anti-TNF antibody (□), or isotype control (■) prior to determination of TNF bioactivity ($N = 4$).

produced TNF, but not LT. Finally, the presence of the TNF gene product was confirmed by PCR, which was used to amplify the predicted 276 bp fragment of the TNF cDNA. The 276 bp fragment was observed in the LPS-stimulated mTALH and the RAW 264 cells.

TNF production resulting from LPS-stimulation of the mTALH may have significant implications related to mTALH function. The mTALH maintains the high medullary interstitial osmolality necessary for the maintenance of the renal countercurrent exchange system. Further, alterations in the capacity of the mTALH to absorb electrolytes independent of water have significant effects on the regulation of extracellular fluid volume, and there is evidence that TNF may be an important mediator/modulator of these mechanisms. For example, sublethal doses of TNF administered to dogs caused a marked polyuria and natriuresis [22]. Indeed, an alteration in ion transport mediated by TNF produced by the mTALH could lead to a natriuretic state, as well as a loss in the hypertonic gradient essential for water reabsorption [22].

TNF has been implicated as one of the major pathological mediators during endotoxemia [23]. This is supported by evidence that TNF levels rise significantly during endotoxemia, and that anti-TNF antibodies have been shown to prevent the toxic effects of gram-negative bacteria. An increase in the production of prostaglandins during septic shock also may mediate some of the symptoms of septicemia. For example, administration of indomethacin to TNF-treated rats prevented the toxic effects associated with shock [24]. In fact, TNF has

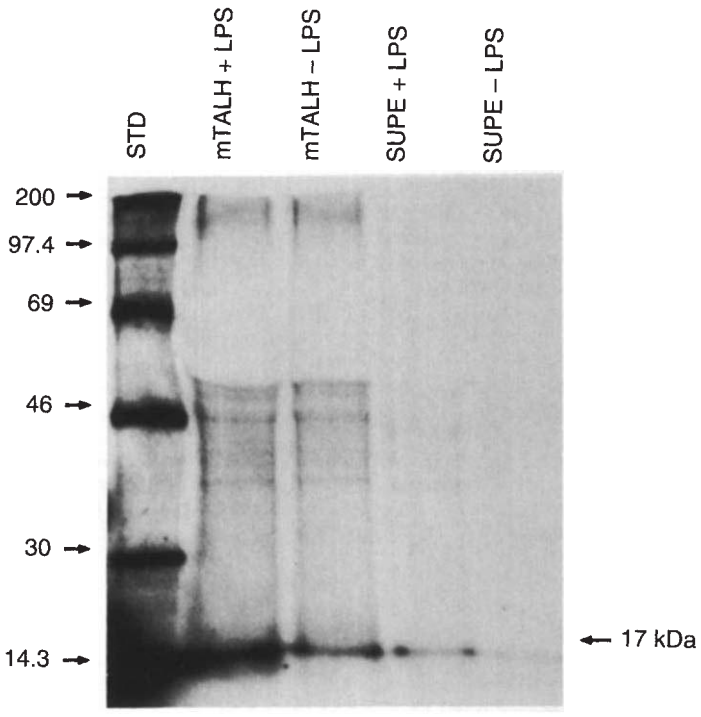

Fig. 6. *De novo synthesis of TNF.* mTALH tubules were metabolically radiolabeled with 200 μCi/ml of ^{35}S-methionine/^{35}S-cysteine in the presence or absence of LPS for 24 hours. Supernatants (SUPE) and tubule lysates (mTALH) were immunoprecipitated with a polyclonal anti-TNF antibody and labeled proteins were analyzed by separation on a 15% SDS-PAGE and exposure for 48 hours to Kodak X-Omat film.

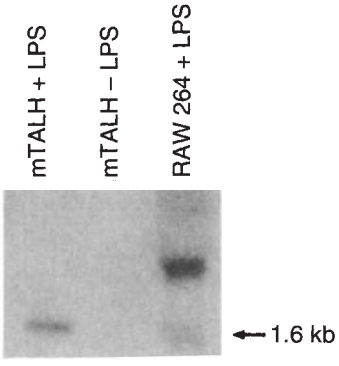

Fig. 7. *Accumulation of TNF mRNA.* mTALH tubules were incubated in the presence or absence of LPS for 24 hours, and total RNA was extracted and analyzed by Northern blot analysis using a radiolabeled cDNA probe for TNF. LPS-stimulated RAW 264 cells were used as a positive control. This is a representative figure from four separate experiments.

been shown to stimulate AA metabolism in several systems, including the kidney [25, 26]. This increase in AA metabolism is related to TNF-mediated transcription of the PLA_2 gene and the subsequent increase of AA release from cellular membranes [27–29], and transcription of the prostaglandin H synthase 2 gene, which encodes an isoform of cyclooxygenase, commonly referred to as COX-2 [30]. Thus, some actions of TNF may be mediated via metabolites of AA. Indeed, this appeared to be the case in the mTALH where the TNF-mediated inhibition of ^{86}Rb

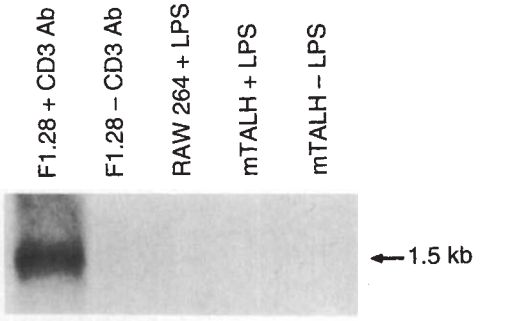

Fig. 8. *Accumulation of LT mRNA.* mTALH tubules were incubated in the presence or absence of LPS for 24 hours, total RNA was analyzed by Northern blot analysis using a radiolabeled cDNA probe for LT. RAW 264 cells were used as a negative control, and F1.28 T cells stimulated with anti-CD3 antibody were used as a positive control. This is a representative figure from four separate experiments.

uptake was diminished by treatment with indomethacin [12]. Exogenous TNF, as well as mTALH-derived TNF formed after challenge with LPS, inhibited ^{86}Rb uptake via a prostaglandin-dependent mechanism. In each case, the concentrations of PGE_2 were significantly higher in the LPS-stimulated mTALH compared to unstimulated mTALH [12]. Thus, the effect of TNF on ion transport in the mTALH may be related to induction of cyclooxygenase, with subsequent PGE_2 synthesis. In fact, PGE_2 has been found to directly inhibit NaCl reabsorption in cultured mTALH cells and in rat mesangial cells, and medullary interstitial NaCl concentrations were increased by inhibition of prostaglandin synthesis [25, 31, 32]. Production of TNF by the mTALH and the effects of TNF on mTALH function suggest that TNF may act in an autocrine manner to affect ion transport in the mTALH via a prostaglandin-dependent mechanism. The concentration of TNF produced by mTALH tubules was approximately 75 nM. This was similar to the concentration of TNF produced by cultured mTALH cells which produced approximately 5 nM TNF when stimulated with LPS for 24 hours [12]. Thus, the levels of TNF produced by LPS-stimulated mTALH tubules were similar to concentrations of exogenous TNF which were shown to affect mTALH function, and support the hypothesis that TNF can act as an endogenous regulator of mTALH function.

It is important to recognize that as the hypotension associated with exposure to endotoxin progresses, renal blood flow is increasingly compromised and may result in end organ damage. Since the mTALH actively transports ions across its membrane, this nephron segment is highly oxygen-dependent. As the mTALH has a limited capacity for anaerobic metabolism, this segment of the nephron can maintain functional integrity only for short periods in the absence of oxygen [33]. Early intervention with agents which would serve to attenuate the induction of TNF following exposure to endotoxin may serve to limit or delay renal damage. However, in cases of acute and chronic inflammation, these effects may be manifested over a longer duration of time. In either case, intervention subsequent to TNF production, may have renal-protective effects.

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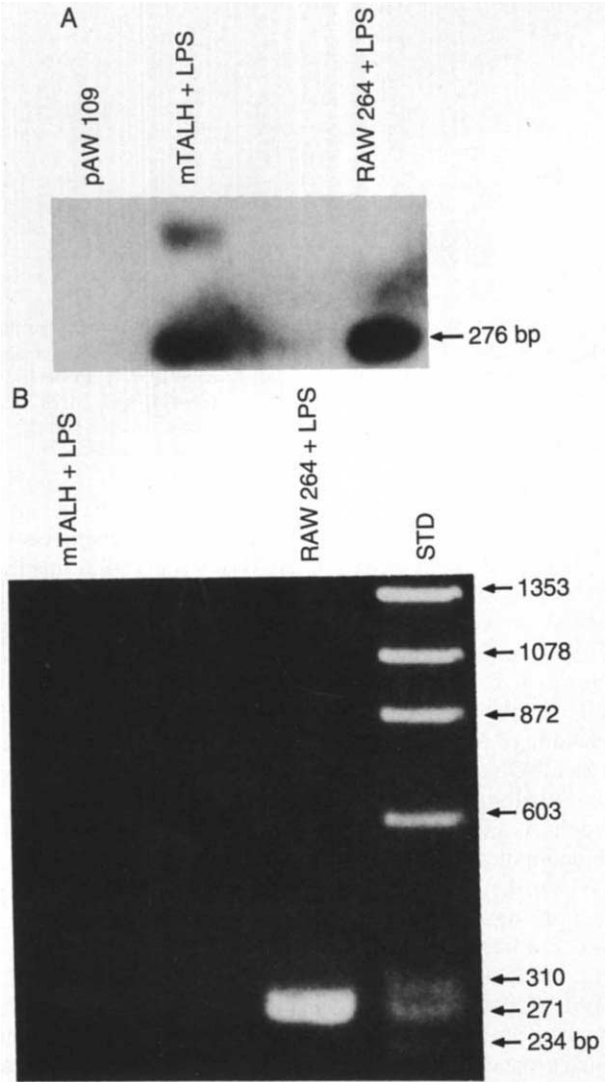

Fig. 9. *PCR analysis of the TNF gene.* Total RNA was extracted from LPS-stimulated mTALH tubules and RAW 264 cells (positive control). The RNA was reverse transcribed, and DNA was amplified by PCR. The TNF gene was detected by: (**A**) Southern blot analysis and (**B**) ethidium bromide staining of DNA.

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References

1. Vassalli P: The pathophysiology of tumor necrosis factors. *Annu Rev Immunol* 10:411–452, 1992
2. Yard BA, Daha MR, Kooymans-Couthino M, Bruijn JA, Paape ME, Schrama E, Van Es LA, van der Woude FJ: IL-1α stimulated TNFα production by cultured human proximal tubular epithelial cells. *Kidney Int* 42:383–389, 1992
3. Baud L, Oudinet J-P, Bens M, Noe L, Peraldi M-N, Rondeau E, Etienne J, Ardaillou R: Production of tumor necrosis factor by rat mesangial cells in response to bacterial lipopolysaccharide. *Kidney Int* 35:1111–1118, 1989
4. Chung IY, Benveniste EN: Tumor necrosis factor-α production by astrocytes: Induction by lipopolysaccharide, IFN-γ, and IL-1β^1. *J Immunol* 144:2999–3007, 1990
5. Jevnikar AM, Brennan DC, Singer GG, Heng JE, Malinski W, Wuthrich RP, Glimcher LH, Kelley VE: Stimulated kidney tubular epithelial cells express membrane associated and secreted TNFα. *Kidney Int* 40:203–211, 1991
6. Ulich TR, Guo K, Del Castillo J: Endotoxin-induced cytokine gene expression *in vivo*. *Am J Pathol* 134:11–14, 1989
7. Sakr M, Zetti G, McClain C, Gavaler J, Nalesnik M, Todo S, Starzl T, Van Thiel D: The protective effect of FK506 pretreatment against renal ischemia/reperfusion injury in rats. *Transplantation* 53:987–991, 1992
8. Hession C, Decker JM, Sherblom AP, Kumar S, Yue CC, Mattaliano RJ, Tizard R, Kawashima E, Schmeissner U, Heletky S, Chow EP, Burne CA, Shaw A, Muchmore AV: A renal ligand for lymphokines. *Science* 237:1479–1484, 1987
9. Ishikura H, Takahashi C, Kanagawa K, Hirata H, Imai K, Yoshiki T: Cytokine regulation of ICAM-1 expression on human renal tubular epithelial cells *in vitro*. *Transplantation* 51.1272–1275, 1991
10. Caverzasio J, Rizzoli R, Dayer J-M, Bonjour J-P: Interleukin-1 decreases renal sodium reabsorption: Possible mechanism of endotoxin-induced natriuresis. *Am J Physiol* 252:F943–F946, 1987
11. Zeidel ML, Brady H, Kohan DE: Interleukin-1 inhibition of Na^+-K^+ATPase in inner medullary collecting ducts cells: Role of PGE_2. *Am J Physiol* 261:F1013–F1016, 1991
12. Escalante BA, Ferreri NR, Dunn CE, McGiff JC: Cytokines affect ion transport in primary cultured thick ascending limb of Henle's loop cells. *Am J Physiol* (in press)
13. Trinh-Trang-Tan M-M, Bouby N, Coutaud C, Bankir L: Quick isolation of rat medullary thick ascending limbs: Enzymatic and metabolic characterization. *Pflügers Arch* 407:228–234, 1986
14. Kikeri D, Azar S, Sun A, Zeidel ML, Hebert SC: Na-H antiporter and Na(HCO) symporter regulate intracellular pH in mouse medullary thick limbs of Henle. *Am J Physiol* 258:F445–F456, 1990
15. Bradford MM: A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72:246–254, 1976
16. Ruddle NH, Bergman CM, McGrath KM, Lingenheld EG, Grunnet ML, Padula SJ, Clark RB: An antibody to lymphotoxin and tumor necrosis factor prevents transfer of experimental allergic encephalomyelitis. *J Exp Med* 172:1193–1200, 1990
17. Keogh C, Fong Y, Marano MA, Seniuk S, He W, Barber A, Minei JP, Felsen D, Lowry SF, Moldawer LL: Identification of a novel tumor necrosis factor α/cachectin from the livers of burned and infected rats. *Arch Surg* 125:79–85, 1990
18. Li C-B, Gray PW, Lin P-F, McGrath KM, Ruddle FH, Ruddle NH: Cloning and expression of murine lymphotoxin cDNA. *J Immunol* 138:4496–4501, 1987
19. Ferreri NR, Sarr T, Askenase PW, Ruddle NH: Molecular regulation of tumor necrosis factor-α and lymphotoxin production in T cells. *J Biol Chem* 267:9443–9449, 1992.
20. Chamberlin ME, Mandel LJ: Substrate support of medullary thick ascending limb oxygen consumption. *Am J Physiol* 251:F758–F763, 1986
21. Wuthrich RP, Glimcher LH, Yui MA, Jevnikar AM, Dumas SE, Kelley VE: MHC class II, antigen presentation and tumor necrosis factor in renal tubular epithlial cells. *Kidney Int* 37:783–792, 1990
22. Van-Lanschot JJ, Mealy K, Jacobs DO, Evan DO, Wilmore DW: Splenectomy attenuates the inappropriate diruesis associated with tumor necrosis factor administration. *Surg Gynecol Obstet* 172:293–297, 1991
23. Beutler B, Greenwald D, Hulmes JD, Chang M, Pan Y-CE, Mathison J, Ulevitch R, Cerami A: Identity of tumour necrosis factor and the macrophage-secreted factor cachetin. *Nature* 316: 552–554, 1985
24. Kettelhut IC, Fiers W, Goldberg AL: The toxic effects of

tumor necrosis factor *in vivo* and their prevention by cyclooxygenase inhibitors. *Proc Natl Acad Sci USA* 84:4273–4277, 1987
25. BAUD L, PEREZ J, FRIEDLANDER G, ARDAILLOU R: Tumor necrosis factor stimulates prostanglandin production and cyclic AMP levels in rat cultured mesangial cells. *FEBS Lett* 239:50–54, 1988
26. ROSSI V, BREVIARIO F, GHEZZI P, DEJANA E, MANTOVANI A: Prostacyclin synthesis induced in vascular cells by interleukin-1. *Science* 229:174–176, 1985
27. KRONKE M, SCHUTZE S, SCHEURICH P, PFIZENMAIER K: TNF signal transduction and TNF-responsive genes. *Immunol Ser* 56: 189–216, 1992
28. CLARK MA, CHEN M-J, CROOKE ST, BOMALASKI JS: Tumour necrosis factor (cachectin) induces phospholipase A_2 activity and synthesis of a phospholipase A_2-activating protein in endothelial cells. *Biochem J* 250:125–132, 1988
29. O'SULLIVAN MG, CHILTON FH, HUGGINS EM, MCCALL CE: Lipopolysaccharide priming of alveolar macrophages for enhanced synthesis of prostanoids involves induction of a novel prostaglandin H synthase. *J Biol Chem* 267:14547–14550, 1992
30. COYNE DW, NICKOLS M, BERTRAND W, MORRISON AR: Regulation of mesangial cell cyclooxygenase synthesis by cytokines and glucocorticoids. *Am J Physiol* 263:F97–F102, 1992
31. CULPEPPER RM, ANDREOLI TE: Interactions among prostaglandin E_2, antidiurectic hormone, and cyclic adenosine monophosphate in modulating Cl^- absorption in single mouse medullary thick ascending limbs of Henle. *J Clin Invest* 71:1588–1601, 1983
32. HERBERT SC, ANDREOLI TE: Control NaCl transport in the thick ascending limb. *Am J Physiol* 246:F745–F756, 1984
33. GUDER WG, Ross BD: Enzyme distribution along the nephron. *Kidney Int* 26:101–111, 1984